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(54) Title: WATER STRESS OR SALT STRESS TOLERANT TRANSGENIC CEREAL PLANTS

(57) Abstract

The present invention relates to a transgenic cereal plant and to a cereal plant cell or protoplast transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance to the plant. Another aspect of the present invention is a method of conferring water stress or salt stress tolerance to a cereal plant including transforming a cereal plant cell or protoplast with a nucleic acid encoding an enzyme for proline biosynthesis. The present invention also relates to a method of increasing tolerance of a cereal plant to water stress or salt stress conditions, the method including increasing levels of an enzyme for proline biosynthesis in the cereal plant. Yet another aspect of the present invention is a transgenic cereal plant transformed with a plasmid that confers water stress or salt stress tolerance to the cereal plant.

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WATER STRESS OR SALT STRESS TOLERANT TRANSGENIC CEREAL PLANTS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/090.561. filed June 24. 1998.

FIELD OF THE INVENTION

The present invention relates to transgenic cereal plants which are transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance to the plant and a method of increasing or conferring water stress or salt stress tolerance to a cereal plant.

BACKGROUND OF THE INVENTION

Environmental stresses. such as drought. increased salinity of soil. and extreme temperature. are major factors in limiting plant growth and productivity. The worldwide loss in yield of three major cereal crops. rice. maize (corn). and wheat due to water stress (drought) has been estimated to be over ten billion dollars annually. Salt stress and drought stress are the two most important abiotic stresses. Of the 4.870 million hectares of agricultural land in the world. 930 million (19% of total) are salt-affected areas (FAO Quarterly Bulletin of Statistics, Vol. 9 ¾ (1996)). Moderate levels of salt content in the soil (such as 50 mM) cause a substantial decrease in the yield of crops. High levels of salt in the soil (higher than 100 or 150 mM) are not at all suitable for planting most cereal crops. Approximately 5.2% of the agricultural lands are under drought stress (FAO Quarterly Bulletin of Statistics, Vol. 9 ¾ (1996)). and the loss of crop yield is also very significant.

In practical terms. rice is the most important crop because a high percentage of the world's population depends on it for their staple food. Together with wheat and corn. these three cereal crops constitute the major source of food and calories to feed the people. With an increase in population and a decrease in arable land. there is a real possibility of a food shortage by the year 2030. Therefore. it is essential to fully utilize plant biotechnology to improve plants and produce more food.

Breeding of stress-tolerant crop cultivars represents a promising strategy to tackle these problems (Epstein et al.. "Saline Culture of Crops: A Genetic Approach."

Science, 210:399-404 (1980)). However, conventional breeding is a slow process for generating crop varieties with improved tolerance to stress conditions. Limited germplasm resources for stress tolerance and incompatibility in crosses between distantly related plant species are additional problems encountered in conventional breeding. Recent progress in plant genetic transformation and availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (Tarczynski et al., "Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol." Science, 259:508-510 (1993); Pilon-Smits et al., "Improved Performance of Transgenic Fructan-Accumulating Tobacco Under Drought Stress." Physiol. Plant, 107:125-130 (1995)). Transformation of cereal plants with agronomically useful genes that increase tolerance to abiotic stress is one important way to minimize yield loss. For example, it would be highly desirable to produce transgenic rice plants that can give reasonable yield when grown in marginal or waste lands that contain relatively high levels of salt, such as 100-150 mM, in the soil.

Characterization and cloning of plant genes that confer stress tolerance remains a challenge. Genetic studies revealed that tolerance to drought and salinity in some crop varieties is principally due to additive gene effects (Akbar et al., "Breeding For Soil Stress." *In* Progress in Rainfed Lowland Rice, International Rice Research Institute, manila, Philippines, pp. 263-272 (1986); Akbar et al., "Genetics of Salt Tolerance in Rice." *In* Rice Genetics, International Rice Research Institute, Manila, Philippines, pp. 399-409 (1986)). However, the underlying molecular mechanism for the tolerance has never been revealed. Physiological and biochemical responses to high levels of ionic or nonionic solutes and decreased water potential have been studied in a variety of plants. Based on accumulated experimental observations and theoretical consideration, one suggested mechanism that may underlie the adaptation or tolerance of plants to osmotic stresses is the accumulation of compatible, low molecular weight osmolytes such as sugar alcohols, special amino acids, and glycine betaine (Greenway et al., "Mechanisms of Salt Tolerance in Nonhalophytes." Annu. Rev. Plant Physiol., 31: 149-190 (1980); Yancey et al., "Living With Water Stress: Evolution of Osmolyte System." Science, 217: 1214-1222 (1982)). In particular, proline level is known to increase in a number of plants and bacteria under drought or salt stress. Recently, a transgenic study has demonstrated that accumulation of the sugar alcohol mannitol in transgenic tobacco conferred protection against salt stress (Tarczynski et al., "Stress Protection of Transgenic Tobacco by

Production of the Osmolyte Mannitol." Science. 259:508-510 (1993)). Two recent studies using a transgenic approach have demonstrated that metabolic engineering of the glycine betaine biosynthesis pathway is not only possible but also may eventually lead to production of stress-tolerant plants (Holmstrom et al., "Production of the *Escherichia coli* Betaine-Aldehyde Dehydrogenase, An Enzyme Required for the Synthesis of the Osmoprotectant Glycine Betaine, in Transgenic Plants." Plant J., 6:749-758 (1994); Rathinasabapathi et al., "Metabolic Engineering of Glycine Betaine Synthesis: Plant Betaine Aldehyde Dehydrogenases Lacking Typical Transit Peptides are Targeted to Tobacco Chloroplasts Where they Confer Betaine Aldehyde Resistance." Planta. 193:155-162 (1994)).

In addition to metabolic changes and accumulation of low molecular weight compounds, a large set of genes is transcriptionally activated which leads to accumulation of new proteins in vegetative tissue of plants under osmotic stress conditions, including the late embryogenesis abundant (LEA) family, dehydrines, and COR47 (Skriver et al., "Gene Expression in Response to Abscisic Acid and Osmotic Stress." Plant Cell. 2:503-512 (1990); Chandler et al., "Gene Expression Regulated by Abscisic Acid and its Relation to Stress Tolerance." Annu. Rev. Plant Physiol. Plant Mol. Biol., 45:113-141 (1994)). The expression levels of a number of genes have been reported to be correlated with desiccation, salt, or cold tolerance of different plant varieties of the same species. It is generally assumed that stress-induced proteins might play a role in tolerance, but the functions of many stress-responsive genes are unknown.

Elucidating the function of these stress-responsive genes and enzymes involved in the biosynthesis of stress-induced osmolytes will not only advance the understanding of plant adaptation and tolerance to environmental stresses, but also may provide important information for designing new strategies for crop improvement (Chandler et al., "Gene Expression Regulated by Abscisic Acid and its Relation to Stress Tolerance." Annu. Rev. Plant Physiol. Plant Mol. Biol., 45:113-141 (1994)).

Several genes that encode key enzymes involved in the biosynthesis of specific osmolytes (such as mannitol, proline, or glycine betaine) have been introduced into tobacco cells. The regenerated transgenic tobacco plants showed partial tolerance to drought and to salt stress (Tarczynski et al., "Stress Protection of Transgenic Tobacco by Production of Osmotic Mannitol." Science. 259:508-510 (1993); Kishor et al., "Overexpression of Δ^1-pyrroline-5-carboxylate Synthetase Increases Proline Production

and Confers Osmotolerance in Transgenic Plants." Plant Physiol., 108:1387-1394 (1995); Lilius et al., "Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase." Biotech., 14:177-180 (1966)). However, only transgenic tobacco was used for these studies, and similar work on producing stress-tolerant cereal crop plants has not been carried out. It is not clear whether these genes in transgenic cereal plants will enhance salt or drought tolerance since the physiology of dicot plants, such as tobacco, is very different from monocots, such as cereal plants. Thus, only experimentation using cereal crop plants can provide the answer.

The present invention is directed to overcoming the above-noted deficiencies in the prior art.

SUMMARY OF THE INVENTION

The present invention relates to a transgenic cereal plant transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance to the plant.

The present invention also relates to a cereal plant cell or protoplast transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance on a cereal plant regenerated from said cereal plant cell or protoplast.

Another aspect of the present invention is a method of conferring water stress or salt stress tolerance to a cereal plant including transforming a cereal plant cell or protoplast with a nucleic acid encoding an enzyme for proline biosynthesis.

The present invention also relates to a method of increasing tolerance of a cereal plant to water stress or salt stress conditions, the method including increasing levels of an enzyme for proline biosynthesis in the cereal plant.

The present invention allows the production of cereal plants with increased tolerance to water stress (drought) and salt stress. Thus, an enzyme for proline biosynthesis can be used as a molecular tool for genetic crop improvement by conferring stress tolerance.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a transgenic cereal plant transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance to the plant.

Suitable nucleic acids encoding an enzyme for proline biosynthesis include the *P5CS* gene of mothbean and a feedback-inhibition insensitive mutant, *P5CS*-129A, of the *P5CS* gene. The sequence of the *P5CS* gene can be found in Kishor et al., "Overexpression of Δ^1-pyrroline-5-carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants," Plant Physiol., 108:1387-1394 (1995), which is hereby incorporated by reference, and the sequence of the *P5CS*-129A mutant gene can be found in Zhang et al., "Removal of Feedback Inhibition of *P5CS* in Plants," J. Biol. Chem., 270:20491-20496 (1995), which is hereby incorporated by reference.

Cereal which can be transformed in accordance with the subject invention are members of the family *Gramineae* (also known as *Poaceae*), and include rice (genus *Oryza*), wheat, maize (corn), barley, oat, sorghum, and millet. Preferably, the cereal is rice, wheat, or corn, and most preferably the cereal is rice. Many species of cereals can be transformed, and within each species the numerous subspecies and varieties can be transformed. For example, within the rice species is subspecies Indica rice (*Oryza sativa* ssp. Indica), which includes the varieties IR36, IR64, IR72, Pokkali, Nona Bokra, KDML105, Suponburi 60, Suponburi 90, Basmati 385, and Pusa Basmati 1. Another rice subspecies is Japonica, which includes Nipponbere, Kenfeng and Tainung 67. Examples of suitable maize varieties include A188, B73, VA22, L6, L9, K1, 509, 5922, 482, HNP, and IGES. Examples of suitable wheat varieties include Pavon, Anza, Chris, Coker 983, FLA301, FLA302, Fremont and Hunter.

Having identified the cereal plant of interest, plant cells suitable for transformation include immature embryos, calli, suspension cells, and protoplasts. It is particularly preferred to use suspension cells and immature embryos.

These cereal plant cells are transformed with a nucleic acid, which could be RNA or DNA and which is preferably cDNA, encoding an enzyme for proline biosynthesis. The nucleic acid can be biologically isolated or synthetic. In the following Examples, a key enzyme for proline biosynthesis, Δ^1-pyrroline-5-carboxylate synthase (P5CS), is encoded by the *P5CS* gene of mothbean. However, other genes encoding an

enzyme for proline biosynthesis, including a feedback-inhibition insensitive mutant of the *P5CS* gene, *P5CS*-129A, can also be utilized.

Transformation of plant cells can be accomplished by using a plasmid. The plasmid is used to introduce the nucleic acid encoding an enzyme for proline biosynthesis into the plant cell. Accordingly, a plasmid preferably includes DNA encoding an enzyme for proline biosynthesis inserted into a unique restriction endonuclease cleavage site. Heterologous DNA, as used herein, refers to DNA not normally present in the particular host cell transformed by the plasmid. DNA is inserted into the vector using standard cloning procedures readily known in the art. This generally involves the use of restriction enzymes and DNA ligases, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), which is hereby incorporated by reference. The resulting plasmid which includes nucleic acid encoding an enzyme for proline biosynthesis can then be used to transform a host cell, such as an *Agrobacterium* and/or a plant cell. (See generally, Plant Molecular Biology Manual, 2nd Edition, Gelvin et al., Eds., Kluwer Academic Press, Dordrecht, Netherlands (1994), which is hereby incorporated by reference).

For plant transformation, the plasmid preferably also includes a selectable marker for plant transformation. Commonly used plant selectable markers include the hygromycin phosphotransferase (*hpt*) gene, the phosphinothricin acetyl transferase gene (*bar*), the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS), neomycin 3'-O-phosphotransferase gene (*npt* II), or acetolactate synthase gene (ALS). Information on these selectable markers can be found in Bowen, "Markers for Plant Gene Transfer" in Transgenic Plants, Kung et al., Eds., Vol. 1, pp. 89-123, Academic Press, NY (1993), which is hereby incorporated by reference.

The plasmid preferably also includes suitable promoters for expression of the nucleic acid encoding an enzyme for proline biosynthesis and for expression of the marker gene. The cauliflower mosaic virus 35S promoter is commonly used for plant transformation, as well as the rice actin 1 gene promoter. In plasmid pJS102 used in the following Examples, the nucleic acid encoding an enzyme for proline biosynthesis is under the control of the constitutive rice actin 1 gene promoter and the marker gene (*bar*) is under control of the cauliflower mosaic virus 35S promoter. Other promoters useful for plant transformation with an enzyme for proline biosynthesis include those from the

genes encoding ubiquitin and proteinase inhibitor II (PINII), as well as stress-induced promoters (such as the HVA1 gene promoter of barley, an abscisic acid (ABA)-inducible promoter, such as ABRC1 from barley linked to a rice Act-100 minimal promoter, and a HVA22 intron).

The plasmid designated pJS112 has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, under ATCC Accession No. ____ on June 17, 1999.

For plant transformation, the plasmid also preferably includes a nucleic acid molecule encoding a 3' terminator such as that from the 3' non-coding region of genes encoding a proteinase inhibitor, actin, or nopaline synthase (nos).

Other suitable plasmids for use in the subject invention can be constructed. For example, genes encoding an enzyme for proline biosynthesis other than the *P5CS* gene of mothbean could be ligated into plasmid JS109 after use of restriction enzymes to remove the *P5CS* gene. Other promoters could replace the actin 1 gene promoter present in plasmid JS102. Alternatively, other plasmids in general containing genes encoding an enzyme for proline biosynthesis under the control of a suitable promoter, with suitable selectable markers, can be readily constructed using techniques well known in the art.

Having identified the plasmid, one technique of transforming cereal plant cells with a gene which encodes for an enzyme for proline biosynthesis is by contacting the plant cell with an inoculum of an *Agrobacterium* bacteria transformed with the plasmid comprising the gene that encodes for the enzyme for proline biosynthesis. Generally, this procedure involves inoculating the plant cells with a suspension of the transformed bacteria and incubating the cells for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants.

In inoculating the cells of cereal plants with *Agrobacterium* according to the subject invention, the bacteria must be transformed with a vector which includes a gene encoding for an enzyme for proline biosynthesis.

Plasmids, suitable for incorporation in *Agrobacterium*, which include a gene encoding for an enzyme for proline biosynthesis, contain an origin of replication for replication in the bacterium *Escherichia coli*, an origin of replication for replication in the bacterium *Agrobacterium tumefaciens*, T-DNA right border sequences for transfer of genes to plants, and marker genes for selection of transformed plant cells. Particularly preferred is the vector pBI121 which contains a low-copy RK2 origin of replication, the neomycin phosphotransferase (nptII) marker gene with a nopaline synthase (NOS) promoter and a NOS 3' polyadenylation signal. T-DNA plasmid vector pBI121 is available from Clonetech Laboratories, Inc., 4030 Fabian Way, Palo Alto, California 94303. A gene encoding for an enzyme for proline biosynthesis is inserted into the vector to replace the beta-glucuronidase (GUS) gene.

Typically, *Agrobacterium* spp. are transformed with a plasmid by direct uptake of plasmid DNA after chemical and heat treatment, as described by Holsters et al. "Transfection and Transformation of *Agrobacterium tumefaciens*," Mol. Gen. Genet., 163:181-187 (1978), which is hereby incorporated by reference: by direct uptake of plasmid DNA after electroporation, as described by Shen et al., "Efficient Transformation of *Agrobacterium* spp. by High Voltage Electroporation," Nucleic Acids Research, 17:8385 (1989), which is hereby incorporated by reference: by triparental conjugational transfer of plasmids from *Escherichia coli* to *Agrobacterium* mediated by a Tra+ help strain as described by Ditta et al., "Broad Host Range DNA Cloning System for Gram-negative Bacteria: Construction of a Gene Bank of *Rhizobium meliloti*," Proc. Natl. Acad. Sci. USA, 77:7347-7351 (1981), which is hereby incorporated by reference: or by direct conjugational transfer from *Escherichia coli* to *Agrobacterium* as described by Simon et al., "A Broad Host Range Mobilization System for *in vivo* Genetic Engineering: Transposon Mutagenesis in Gram-negative Bacteria," Biotechnology, 1:784-791 (1982), which is hereby incorporated by reference.

Another method for introduction of a plasmid containing nucleic acid encoding an enzyme for proline biosynthesis into a plant cell is by transformation of the plant cell nucleus, such as by particle bombardment. As used throughout this application, particle bombardment (also known as biolistic transformation) of the host cell can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by

reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the plasmid can be introduced into the cell by coating the particles with the plasmid containing the heterologous DNA. Alternatively, the target cell can be surrounded by the plasmid so that the plasmid is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the plasmid and heterologous DNA) can also be propelled into plant cells.

A further method for introduction of the plasmid into a plant cell is by transformation of plant cell protoplasts (stable or transient). Plant protoplasts are enclosed only by a plasma membrane and will therefore take up macromolecules like heterologous DNA. These engineered protoplasts can be capable of regenerating whole plants. Suitable methods for introducing heterologous DNA into plant cell protoplasts include electroporation and polyethylene glycol (PEG) transformation. As used throughout this application, electroporation is a transformation method in which, generally, a high concentration of plasmid DNA (containing heterologous DNA) is added to a suspension of host cell protoplasts and the mixture shocked with an electrical field of 200 to 600 V/cm. Following electroporation, transformed cells are identified by growth on appropriate medium containing a selective agent.

As used throughout this application, transformation encompasses stable transformation in which the plasmid is integrated into the plant chromosomes.

In the Examples which follow, rice has been transformed using biolistic transformation. Other methods of transformation have also been used to successfully transform rice plants, including the protoplast method (for a review, see Cao et al., "Regeneration of Herbicide Resistant Transgenic Rice Plants Following Microprojectile-Mediated Transformation of Suspension Culture Cells," Plant Cell Rep., 11:586-591 (1992), which is hereby incorporated by reference), and the *Agrobacterium* method (Hiei et al., "Efficient Transformation of Rice (*Oryza sativa* L.) Mediated by *Agrobacterium* and Sequence Analysis of the Boundaries of the T-DNA," The Plant Journal, 6:271-282 (1994), which is hereby incorporated by reference). Biolistic transformation has also been used to successfully transform maize (for a review, see Mackey et al., "Transgenic Maize," *In* Transgenic Plants, Kung et al., Eds., vol. 2, pp. 21-33 (1993), which is hereby

incorporated by reference) and wheat (see U.S. Patent No. 5.405.765 to Vasil et al.. which is hereby incorporated by reference).

Once a cereal plant cell or protoplast is transformed in accordance with the present invention. it is regenerated to form a transgenic cereal plant. Generally. regeneration is accomplished by culturing transformed cells or protoplasts on medium containing the appropriate growth regulators and nutrients to allow for the initiation of shoot meristems. Appropriate antibiotics are added to the regeneration medium to inhibit the growth of *Agrobacterium* or other contaminants and to select for the development of transformed cells or protoplasts. Following shoot initiation, shoots are allowed to develop in tissue culture and are screened for marker gene activity.

In suitable transformation methods. the cereal plant cell to be transformed can be *in vitro* or *in vivo*. i.e..the cereal plant cell can be located in a cereal plant.

The invention also provides seed produced by the transgenic cereal plant. The invention is also directed to seed. which upon germination, produces the transgenic cereal plant.

Also encompassed by the present invention are transgenic cereal plants transformed with fragments of the nucleic acids encoding an enzyme for proline biosynthesis of the present invention. Suitable fragments capable of conferring water stress or salt stress tolerance to cereal plants can be constructed by using appropriate restriction sites. A fragment refers to a continuous portion of the encoding molecule for an enzyme for proline biosynthesis that is less than the entire molecule.

Non-essential nucleotides could be placed at the 5' and/or 3' ends of the fragments (or the full length molecules encoding an enzyme for proline biosynthesis) without affecting the functional properties of the fragment or molecule (i.e. in increasing water stress or salt stress tolerance). For example. the nucleotides encoding an enzyme for proline biosynthesis may be conjugated to a signal (or leader) sequence at the N-terminal end (for example) of the enzyme for proline biosynthesis which co-translationally or post-translationally directs transfer of the enzyme for proline biosynthesis. The nucleotide sequence may also be altered so that the encoded enzyme is conjugated to a linker or other sequence for ease of synthesis. purification. or identification of the enzyme.

The present invention also relates to a cereal plant cell or protoplast transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers

water stress or salt stress tolerance on a cereal plant regenerated from said cereal plant cell or protoplast. Once transformation has occurred, the cereal plant cell or protoplast can be regenerated to form a transgenic cereal plant.

Preferably, the nucleic acid encoding an enzyme for proline biosynthesis is controlled by a strong promoter to effect maximum expression of an enzyme for proline biosynthesis, or by a stress-induced promoter to effect induction of the promoter in response to stress conditions. In one embodiment, the transgenic cereal plant cell or protoplast or plant is transformed with the nucleic acid encoding the promoter, such as the rice actin 1 gene promoter, by providing a plasmid which includes DNA encoding an enzyme for proline biosynthesis and the promoter.

The transgenic cereal plant cell or protoplast or plant can also be transformed with a nucleic acid encoding a selectable marker, such as the *bar* gene, to allow for detection of transformants, and with a nucleic acid encoding the cauliflower mosaic virus 35S promoter to control expression of the *bar* gene. Other selectable markers include genes encoding EPSPS, nptII, or ALS. Other promoters include those from genes encoding actin 1, ubiquitin, and PINII. These additional nucleic acid sequences can also be provided by the plasmid encoding the enzyme for proline biosynthesis and its promoter. Where appropriate, the various nucleic acids could also be provided by transformation with multiple plasmids.

While the nucleotide sequence referred to herein encodes an enzyme for proline biosynthesis, nucleotide identity to previously sequenced enzymes for proline biosynthesis is not required. As should be readily apparent to those skilled in the art, various nucleotide substitutions are possible which are silent mutations (i.e. the amino acid encoded by the particular codon does not change). It is also possible to substitute a nucleotide which alters the amino acid encoded by a particular codon, where the amino acid substituted is a conservative substitution (i.e. amino acid "homology" is conserved). It is also possible to have minor nucleotide and/or amino acid additions, deletions, and/or substitutions in the enzyme for proline biosynthesis nucleotide and/or amino acid sequences which have minimal influence on the properties, secondary structure, and hydrophilic/hydrophobic nature of the encoded enzymes for proline biosynthesis. These variants are encompassed by the nucleic acid encoding an enzyme for proline biosynthesis according to the subject invention.

The present invention is also directed to a transgenic cereal plant regenerated from the transgenic cereal plant cells or protoplasts, as well as to seed produced by the transgenic cereal plants.

Another aspect of the present invention is a method of conferring water stress or salt stress tolerance to a cereal plant including transforming a cereal plant cell or protoplast with a nucleic acid encoding an enzyme for proline biosynthesis.

In a preferred embodiment, the method further includes regenerating the transformed cereal plant cell or protoplast to form a transgenic cereal plant. The present invention also includes seed produced by the transgenic cereal plant.

The present invention also relates to a method of increasing tolerance of a cereal plant to water stress or salt stress conditions, the method including increasing levels of an enzyme for proline biosynthesis in the cereal plant.

In a preferred embodiment, the plasmid is designated pJS102, pJS107, or pJS112 (See Examples 1 and 2).

EXAMPLES

Example 1 – Construction of the pJS107 Plasmid for Plant Transformation

Plasmid Construction

pJS107 was constructed by isolating a 2.4 kb *Sal*I fragment containing mothbean (*Vigna aconitifolia* L.) *P5CS* cDNA from the plasmid pUbiP5CS (Hu et al., "A Bifunctional Enzyme (Δ^1-pyrroline-5-carboxylate synthetase) Catalyzes the First Two Steps in Pro Biosynthesis in Plants." Proc. Natl. Acad. Sci. USA, 89:9354-9358 (1992), which is hereby incorporated by reference). This DNA fragment was blunted with Klenow DNA polymerase and subcloned into the *Sma*I site of the expression vector pJS104 (Su et al., "Dehydration-Stress-Regulated Transgene Expression in Stably Transformed Rice Plants." Plant Physiol., 117:913-922 (1998), which is hereby incorporated by reference) to create pJS107. In pJS107, the *P5CS*-coding sequence was downstream of a stress-inducible promoter complex (designated as AIPC-ABA inducible promoter complex). AIPC includes a 49 bp ABA-responsive element from the barley *Hva22* gene (Shen et al., "Functional Dissection Of An Abscisic Acid (ABA)-Inducible Gene Reveals Two Independent ABA-Responsive Complexes Each Containing A G-Box And A Novel *Cis*-Acting Element." Plant Cell, 7:295-307 (1995), which is hereby

incorporated by reference). a 180 bp minimum rice actin gene promoter (Su et al., "Dehydration-Stress-Regulated Transgene Expression in Stably Transformed Rice Plants." Plant Physiol., 117:913-922 (1998). which is hereby incorporated by reference). and a *Hva22* intron (Shen et al., "Functional Dissection Of An Abscisic Acid (ABA)-Inducible Gene Reveals Two Independent ABA-Responsive Complexes Each Containing A G-Box And A Novel *Cis*-Acting Element." Plant Cell, 7:295-307 (1995). which is hereby incorporated by reference). pJS107 also contains the *bar* cassette. which was used for selection of transgenic calli and plants in the presence of the herbicide. Bialaphos.

Transformation of Rice Cells with a Mothbean P5CS cDNA (Zhu et al., "Overexpression Of A *P5CS* Gene And Analysis Of Tolerance To Water And Salt Stress In Transgenic Rice." Plant Science 139:41-48 (1998). which is hereby incorporated by reference)

The procedure and media used for the establishment of suspension cells was according to a previously described method (Cao et al., "Assessment Of Rice Genetic Transformation Techniques. In Rice Biotechnology. Toenniessen et al., Eds., CAB International, Oxon, UK, pp. 175-198 (1991); Cao et al., "Regeneration Of Herbicide Resistant Transgenic Rice Plants Following Microprojectile-Mediated Transformation Of Suspension Culture Cells." Plant Cell Rep., 11:586-191 (1992). which are hereby incorporated by reference). Dehusked rice seeds (*Oryza sativa* L. var. Nipponbare) were used for callus induction. Following growth in suspension cultures, pJS107 was introduced into suspension culture cells by the biolistic method. The cells were cultured and selected in KPR medium (Cao et al., "Assessment Of Rice Genetic Transformation Techniques. In Rice Biotechnology. Toenniessen et al., Eds., CAB International, Oxon, UK, pp. 175-198 (1991). which is hereby incorporated by reference) containing 8 mg per liter Bialaphos. The resistant calli were transferred to MS regeneration medium to regenerate into plants. Plants regenerated from the same resistant callus were regarded as clones of the same line. Regenerated plants were transferred into soil and grown in the greenhouse (32°C day/22°C night, with supplemental photoperiod of 10 hours).

Plasmid pJS107 (ABRC1/Act-100 promoter/Hva22 intron/*P5CS* cDNA/Pin2 3'//35S promoter/*bar*/Nos 3') was introduced into rice suspension cells using the biolistic-mediated transformation method.

Regeneration and Analysis of Transgenic Plants

A number of transgenic plants (*Oryza sativa* L.) were generated, and four lines with relatively low transgene copy number were analyzed. The results are shown in Table 1, below.

Table 1. Analysis of Transgenic Rice Plants Transformed With a Mothbean P5CS cDNA.

Plant	Southern Blot Copy Number	P5CS Activity[a]	Proline µg/g Fresh Leaf	Shoot Fresh Weight (g)[b]
Control	0	-	27	0.80
N22	5	++	45	1.22
N52	3		48	0.82
N60	2	+++	71	1.51
N70	2	++++	68	1.90

a P5CS activity was assayed based on the conversion of [^{14}C] glutamate to [^{14}C] proline: TLC separation.

b Eight-week-old plants (4-10 per line) were stressed with no water for 6 days, then water 1 day. Four cycles (28 days).

Thus, this data indicated that transgenic rice plants produced an increased level of the P5CS enzyme activity as well as proline content (measured by using a colorimetric method) in leaves.

Example 2 – Transformation of Rice Calli with a Mothbean *P5CS* cDNA and Comparison of an Inducible vs. Constitutive Proline Synthesis in Transgenic Rice

Plasmid Construction

Three plasmids were constructed. The components of these plasmids are:

pJS102 (with a constitutive promoter): Rice actin 1 promoter/*P5CS* cDNA/Pin 2 3'//35S promoter/*bar*/Nos 3';

pJS112 (with a stress-inducible promoter): ABRC4/Act1-100 promoter/*Hva22* intron/*P5CS* cDNA/Pin2 3'//35S promoter/*bar*/Nos 3'; and

pJS110 (with a constitutive promoter and all components as in pJS112, except that a *uidA* reporter gene is used in place of the *P5CS* cDNA in pJS112).

Transformation of Rice Calli with a Mothbean P5CS cDNA

The preparation of rice calli, transformation procedure, and regeneration of plants were similar to those described in Example 1.

Analysis of Transgenic Plants:

Growth and Stress Treatments of Plants in Soil

Refined and sterilized field soil was used to grow the rice plants in the greenhouse. R_2 seeds were germinated in 1/2 MS medium for 7 days, and the 7-day-old seedlings were transplanted into soil in small pots (8x8 inches) with holes in the bottom (4 to 6 plants per pot). The pots were kept in flat-bottomed trays containing water. The seedlings were grown for an additional 2 weeks, and within the third week, they were tested for Basta resistance. Two Basta-resistant plants with the same plant height per pot were selected for stress treatments. Stress treatments were carried out as follows.

In the first round of stress treatment, water was withheld from the trays for 7 days, and, then, the stressed plants were resupplied with water for 2 days. One or three additional rounds of stress treatments were imposed on the plants. For salt stress, 3-week-old plants were transferred to trays containing 300 mM NaCl solution for 20 days. The NaCl solution was changed every 3 days to maintain a constant concentration of NaCl in the soil. The pots containing stressed plants were transferred back to trays containing tap water to allow the stressed plants to recover and grow without stress for 10 days. After the 10 days of recovery, a second round of salt stress was imposed by using the same concentration of NaCl solution for 10 days. Liquid fertilizer (Peters Excel, N:P:K = 15:5:15, Scotts Professional Co.) mixed with tap water or NaCl solution was applied to the plants weekly.

Growth Performance of Transgenic Plants Under Water Stress Conditions

Since there was no significant difference in growth performance between NT plants and *uidA* plants in seedlings tested, the *uidA* plants (L3) were chosen as more suitable control plants for the following experiment because they also contained *bar* and the same promoter cassette as the *p5cs*-transgenic plants.

Before initial water stress, all the 3-week-old plants including the L3 control plants, were tested for Basta resistance. Healthy, Basta-resistant plants with similar plant height were selected for analyzing growth performance. Under non-stress

conditions in soil, no significant differences were observed between *p5cs*-containing transgenic plants and *SIPC-uidA* control plants in their growth performance during the entire period of the experiment. Upon withholding water from the trays, the absolute water content in the soil decreased from 35% to 12% after 7 days water stress. Following 2 cycles of the water stress, the leaves of *SIPC-uidA* control plants started to turn yellow, and the *Act1-p5cs* plants showed low-growth rate, whereas the *SIPC-p5cs* plants with a stress-inducible promoter showed healthy growth. After 4 cycles of water stress, more severe symptoms, such as leaf chlorosis (in both control and *Act1-p5cs* plants) or death of leaf tips (in control plants only), were found. The *SIPC-p5cs* plants still showed a high rate of growth and less-severe leaf chlorosis. Data in Table 2 (top half) show the average fresh shoot weight and fresh root weight of the plants after 4 cycles of 7 days water stress. The results indicated that under water stress, the *SIPC-p5cs* plants (L5 and L7), which contained a stress-inducible promoter to drive the *p5cs* expression, grew much faster as compared to *Act1-p5cs* plants (L1), which contained a constitutive promoter for driving the *p5cs* expression. The difference between using a stress-inducible promoter and a constitutive promoter was highly significant ($P<0.01$: $t = 5.88$ to 7.64).

Growth Performance of Transgenic Rice Plants Under Salt-Stress Conditions

To create high soil salinity, 300 mM NaCl solution was added to the trays in which the pots were placed. At an early stage (10 d after the initial stress), the control plants (L3) started to wilt and the leaves began to turn yellow, whereas the *p5cs* transgenic plants still showed healthy growth. After 20 days of NaCl stress, the *Act1-P5CS* plants (L1) also started to wilt. Following 10 days of watering to allow recovery and an additional 10 days of 300 mM NaCl stress, more severe damage occurred in both control plants (L3) and *Act1-p5cs* plants. On the contrary, the leaves of *SIPC-p5cs* plants still remained green with a high rate of growth. The average fresh shoot weight and fresh root weight are shown in Table II (bottom half). These values indicated that *SIPC-p5cs* plants (L5 and L7) grew significantly larger ($P<0.01$: $t = 6.03$ to 7.79) under salt-stress conditions than *Act1-p5cs* plants (L1) and control plants (L3), in spite of the finding that the proline level was lower in *SIPC-p5cs* plants. Of the two *SIPC-p5cs* lines, L5 was the better one. In conclusion, stress-inducible transgene expression in *p5cs* plants shows significant advantages over constitutive expression of the *p5cs*-transgene in growth of rice plants under salt- and water-stress conditions.

Table 2. Growth performance of transgenic plants in soil under water-stress or salt-stress conditions

Rice Line	Promoter	Fresh Shoot Wt (mg / plant)	Fresh Root Wt (mg / plant)	Comparison	t Value* in Water-Stress Expt. Shoot Wt	Root Wt
JS110 (L3)	Inducible	300±20 (100)	90±20 (100)	L1:L3	9.54	3.21
JS102 (L1)	Constitutive	550±60 (183)	130±20 (144)	L5:L3	14.22	8.05
JS112 (L5)	Inducible	940±100 (310)	220±30 (224)	L7:L3	4.97	6.22
JS112 (L7)	Inducible	730±60 (243)	170±20 (189)	L1:L5	7.64	5.88

Transgenic Line	Promoter	Fresh Shoot Wt (mg / plant)	Fresh Root Wt (mg / plant)	Comparison	t Value* in NaCl-Stress Expt. Shoot Wt	Root Wt
JS110 (L3)	Inducible	320±40 (100)	70±10 (100)	L1:L3	5.68	4.18
JS102 (L1)	Constitutive	580±100 (181)	110±20 (157)	L1:L5	6.03	7.79
JS112 (L5)	Inducible	1030±140 (322)	240±30 (343)	L5:L3	11.72	11.92
JS112 (L7)	Inducible	870±150 (272)	180±30 (257)	L7:L3	7.83	7.67

* As compared to the t values of *Student's distribution* table. $t_{0.05\,(n=6)}$=2.23 and $t_{0.01(n=6)}$=3.17. All values higher than 3.17 are significant.

Fresh shoot and root weights are in mg/plant. Means±SE represents the averages of 6 plants (Wt). Values in parentheses are the percentages of *p5cs*-transgenic plants compared to control plants (L3). represented by 100. The spread of data within each set of 6 plants was rather small. For example. the actual values for the fresh shoot wt of six JS110 (L3) plants in the water-stress experiment (top half of table) were: 280. 282. 288. 315. 320 and 325: the actual values for the fresh shoot wt of six JS112 (L5) plants were: 840. 845. 860. 1025. 1045 and 1050.

Although the invention has been described in detail for the purpose of illustration. it is understood that such detail is solely for that purpose. and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. A transgenic cereal plant transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance to the plant.

2. A transgenic cereal plant according to claim 1, wherein said cereal plant is a rice plant.

3. A transgenic cereal plant according to claim 1, wherein said nucleic acid encoding an enzyme for proline biosynthesis is the *P5CS* gene of mothbean or a mutant mothbean *P5CS* gene which is insensitive to feedback inhibition by proline.

4. A transgenic cereal plant according to claim 3, wherein the mutant mothbean *P5CS* gene is *P5CS*-129A.

5. A transgenic cereal plant according to claim 1, wherein said transgenic cereal plant includes a nucleic acid encoding a promoter, wherein expression of said nucleic acid encoding said enzyme for proline biosynthesis is controlled by said promoter.

6. A transgenic cereal plant according to claim 5, wherein said promoter is the rice actin 1 gene promoter.

7. A transgenic cereal plant according to claim 1, wherein said transgenic cereal plant includes a nucleic acid encoding a selectable marker.

8. A seed produced by the transgenic cereal plant of claim 1.

9. A seed, which upon germination, produces the transgenic cereal plant of claim 1.

10. A cereal plant cell or protoplast transformed with a nucleic acid encoding an enzyme for proline biosynthesis that confers water stress or salt stress tolerance on a cereal plant regenerated from said cereal plant cell or protoplast.

11. A cereal plant cell or protoplast according to claim 10, wherein said cereal plant cell or protoplast is derived from a rice plant.

12. A cereal plant cell or protoplast according to claim 10, wherein said nucleic acid encoding an enzyme for proline biosynthesis is the *P5CS* gene of mothbean or a mutant mothbean *P5CS* gene which is insensitive to feedback inhibition by proline.

13. A cereal plant cell or protoplast according to claim 12, wherein the mutant mothbean *P5CS* gene is *P5CS*-129A.

14. A cereal plant cell or protoplast according to claim 10, wherein said cereal plant cell or protoplast includes a nucleic acid encoding a promoter, wherein expression of said nucleic acid encoding said enzyme for proline biosynthesis is controlled by said promoter.

15. A cereal plant cell or protoplast according to claim 14, wherein said promoter is the rice actin 1 gene promoter.

16. A cereal plant cell or protoplast according to claim 10, wherein said cereal plant cell or protoplast includes a nucleic acid encoding a selectable marker.

17. A transgenic cereal plant regenerated from the cereal plant cell or protoplast of claim 10.

18. A seed produced by the transgenic cereal plant of claim 17.

19. A method of conferring water stress or salt stress tolerance to a cereal plant comprising:

transforming a cereal plant cell or protoplast with a nucleic acid encoding an enzyme for proline biosynthesis under conditions effective to impart water stress or salt stress tolerance to cereal plants.

20. A method according to claim 19, wherein said cereal plant cell or protoplast is derived from a rice plant.

21. A method according to claim 19, wherein said nucleic acid encoding an enzyme for proline biosynthesis is the *P5CS* gene of mothbean or a mutant mothbean *P5CS* gene which is insensitive to feedback inhibition by proline.

22. A method according to claim 21, wherein the mutant mothbean *P5CS* gene is *P5CS*-129A.

23. A method according to claim 19, wherein said transforming comprises:

propelling particles at said cereal plant cell under conditions effective for the particles to penetrate the cell interior; and

introducing a plasmid comprising the nucleic acid encoding an enzyme for proline biosynthesis into the cell interior.

24. A method according to claim 23, wherein the plasmid is associated with the particles, whereby the plasmid is carried into the cell or protoplast interior together with the particles.

25. A method according to claim 19, wherein said transforming comprises:

contacting tissue of the monocot plant with an inoculum of a bacterium of the genus *Agrobacterium*, wherein the bacterium is transformed with a plasmid comprising the gene that increases tolerance to salt stress and drought stress.

26. A method according to claim 19 further comprising:

regenerating the transformed cereal plant cell or protoplast to form a transgenic cereal plant.

27. A transgenic cereal plant produced by the method of claim 26.

28. A seed produced by the transgenic cereal plant of claim 27.

29. A method of increasing tolerance of a cereal plant to water stress or salt stress conditions, said method comprising:

increasing levels of an enzyme for proline biosynthesis in said cereal plant.

30. A method according to claim 29, wherein said cereal plant is a rice plant.

31. A method according to claim 29, wherein said nucleic acid encoding an enzyme for proline biosynthesis is the *P5CS* gene of mothbean or a mutant mothbean *P5CS* gene which is insensitive to feedback inhibition by proline.

32. A method according to claim 31, wherein the mutant mothbean *P5CS* gene is *P5CS*-129A.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14336

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 1/00; C12N 5/04, 5/10, 15/63, 15/64, 15/82, 15/84, 15/90

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 410, 419, 430, 468, 469, 470; 800/278, 293, 294, 295, 298, 320, 320.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Agricola, Caplus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,639,950 A (VERMA et al) 17 June 1997, col. 3, line 40 to col. 4, line13, col. 5, line 38 to col. 8, line 4, claims.	1, 3, 5, 7-10, 12, 14, 16-19, 21, 25-29, 31 --- 2, 11, 20, 23, 24, 30
Y	ZHANG et al. Removal Of Feedback Inhibition Of Delta-Pyrroline-5-carboxylate Synthetase, A Bifunctional Enzyme Catalyzing The First Two Steps Of Proline Biosynthesis In Plants. J. Biol. Chem. 01 September 1995, Vol. 270, No. 35, pages 20491-20496, especially pages 20492, 20494-20496.	3, 4, 12, 12, 13, 21, 22, 31, 32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14336

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	McELROY et al. Isolation Of An Efficient Actin Promoter For Use In Rice Transformation. Plant Cell. February 1990, Vol. 2, pages 163-171, especially pages 165, 168, 169.	6, 15
Y	RASHID et al. Transgenic Plant Production Mediated By Agrobacterium In Indica Rice. Plant Cell Rep. 1996, Vol. 15, No. 10, pages 727-730, see whole document.	2, 11, 20, 25, 30
Y	CHRISTOU et al. Production Of Transgenic Rice (Oryza sativa L.) Plants From Agronomically Important Indica And Japonica Varieties Via Electric Discharge Particle Acceleration Of Exogenous DNA Into Immature Zygotic Embryos. Biotechnology. October 1991, Vol. 9, pages 957-962, see whole document.	2, 11, 20, 23, 24, 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14336

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 410, 419, 430, 468, 469, 470; 800/278, 293, 294, 295, 298, 320, 320.2